

GABA B receptors

The invention relates to polypeptides which exert the biological activity of GABA B receptors and to nucleic acids encoding these polypeptides, and, in particular, to their use for finding active compounds for crop protection.

BACKGROUND OF THE INVENTION

Gamma-amino-butyric acid (GABA) is the most important inhibitory neurotransmitter in the nervous system of vertebrates and invertebrates. The GABA receptors can be classified into two subfamilies, the GABA A and GABA B receptors. Amongst these, the GABA A receptors are ligand-controlled ion channels, while the GABA B receptors are metabotropic, G-protein-coupled receptors. GABA B receptors affect the release of various neurotransmitters and the activity of ion channels.

GABA B receptors have been studied extensively, in particular in vertebrates. Two subtypes (GABA B1 and GABA B2), which are functionally active as heterodimers, are known here (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998).

In insects, GABA is the most important inhibitory neurotransmitter of the central nervous system. Accordingly, GABA receptors can be detected electrophysiologically on preparations of insect central ganglia. Both the GABA A receptors and the GABA B receptors are the molecular target of important natural and synthetic insecticidally active compounds (Sattelle, 1990; Fukunaga et al., 1999).

The protein sequence of a number of insect GABA A receptors is already known. Thus, the sequences of three different subunits have been described for *Drosophila melanogaster* (French-Constant et al., 1991; Harvey et al., 1994; Henderson et al., 1993).

The provision of insect GABA B receptors is therefore of great practical importance, for example in the search for new insecticides.

A BRIEF SUMMARY OF THE INVENTION

The present invention is therefore based in particular on the object of providing insect GABA B receptors and on assay systems based thereon with a high throughput of test compounds (high throughput screening assays; HTS assays).

5 DETAILED DESCRIPTION OF THE INVENTION

The object is achieved by providing polypeptides which exert at least one biological activity of a GABA B receptor and which comprise an amino acid sequence having at least 70% identity, preferably at least 80% identity, especially preferably at least 90% identity, very especially preferably at least 95% identity, with a sequence of SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6 over a length of at least 20, preferably at least 25, especially preferably at least 30 consecutive amino acids, and very especially preferably over their full lengths.

10 The degree of identity of the amino acid sequences is preferably determined using the program GAP from the package GCG, Version 9.1, with standard settings (Devereux et al., 1984).

20 The term "polypeptides" as used in the present context not only relates to short amino acid chains which are usually termed peptides, oligopeptides or oligomers, but also to longer amino acid chains which are usually termed proteins. It encompasses amino acid chains which can be modified either by natural processes, such as post-translational processing, or by chemical prior-art methods. Such modifications may occur at various sites and repeatedly in a polypeptide, such as, for example, on the peptide backbone, on the amino acid side chain, on the amino and/or the carboxyl terminus. For example, they encompass acetylations, acylations, ADP-ribosylations, amidations, covalent linkages to flavins, haem-moieties, nucleotides or nucleotide derivatives, lipids or lipid derivatives or phosphatidylinositol, cyclizations, disulphide bridge formations, demethylations, cystine formations, formylations, gamma-carboxylations, glycosylations, hydroxylations, iodinations, methylations, 25 myristylations, oxidations, proteolytic processings, phosphorylations, selenylations and tRNA-mediated amino acid additions.

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~~DETAILED DESCRIPTION OF THE INVENTION~~

The polypeptides according to the invention may exist in the form of "mature" proteins or parts of larger proteins, for example as fusion proteins. They can furthermore exhibit secretion or leader sequences, pro-sequences, sequences which allow simple purification, such as multiple histidine residues, or additional stabilizing amino acids.

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The biological activity of the GABA B receptors is preferably achieved by heterodimerization of the polypeptides according to the invention. For example, the polypeptides according to the invention with an amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 4, SEQ ID NO: 2 and SEQ ID NO: 6 or SEQ ID NO: 4 and SEQ ID NO: 6 can gain receptor activity by dimerization.

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The polypeptides according to the invention need not constitute complete receptors, but may also be fragments thereof, as long as they still have at least one biological activity of the complete receptors. Polypeptides which, compared with GABA B receptors, are composed of the polypeptides according to the invention with an amino acid sequence of SEQ ID NO: 2 and SEQ ID NO: 4, which have a 50% higher or reduced activity, are still considered to be in accordance with the invention. The polypeptides according to the invention need not be deducible from *Drosophila melanogaster* GABA B receptors. Polypeptides which are also considered as being in accordance with the invention are those which correspond to the GABA B receptors of, for example, the following invertebrates, or fragments thereof which can still exert the biological activity of these receptors: arthropods, nematodes, molluscs.

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In comparison with the corresponding region of naturally occurring GABA B receptors, the polypeptides according to the invention can have deletions or amino acid substitutions, as long as they still exert at least one biological activity of the complete receptors. Conservative substitutions are preferred. Such conservative substitutions encompass variations, one amino acid being replaced by another amino acid from amongst the following group:

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1. small aliphatic residues, unpolar residues or residues of little polarity: Ala, Ser, Thr, Pro and Gly;
2. polar, negatively charged residues and their amides: Asp, Asn, Glu and Gln;
- 5 3. polar, positively charged residues: His, Arg and Lys;
4. large aliphatic unpolar residues: Met, Leu, Ile, Val and Cys; and
5. aromatic residues: Phe, Tyr and Trp.

Preferred conservative substitutions can be seen from the following list:

Original residue	Substitution
Ala	Gly, Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala, Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Tyr, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

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The term "biological activity of a GABA B receptor" as used in the present context means binding GABA.

5 Preferred embodiments of the polypeptides according to the invention are *Drosophila melanogaster* GABA B receptors which have the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6.

10 Subject-matter of the present invention are also nucleic acids which encode the polypeptides according to the invention.

15 The nucleic acids according to the invention are, in particular, single-stranded or double-stranded deoxyribonucleic acids (DNA) or ribonucleic acids (RNA). Preferred embodiments are fragments of genomic DNA which may contain introns, and cDNAs.

cDNAs which have a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 constitute preferred embodiments of the nucleic acids according to the invention.

20 The present invention also encompasses nucleic acids which hybridize under stringent conditions with sequences of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

25 The term "to hybridize" as used in the present context describes the process during which a single-stranded nucleic acid molecule undergoes base pairing with a complementary strand. Starting from the sequence information disclosed herein, this allows, for example, DNA fragments to be isolated from insects other than *Drosophila melanogaster* which encode polypeptides with the biological activity of GABA B receptors.

30 Preferred hybridization conditions are stated hereinbelow:

Hybridization solution: 6X SSC / 0 % formamide, preferred hybridization solution:
6X SSC / 25 % formamide

Hybridization temperature: 34°C, preferred hybridization temperature: 42°C

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Wash step 1: 2X SSC at 40°C,

Wash step 2: 2X SSC at 45°C; preferred wash step 2: 0.6X SSC at 55°C,
especially preferred wash step 2: 0.3 X SSC at 65°C.

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The present invention encompasses furthermore nucleic acids which have at least 70% identity, preferably at least 80% identity, especially preferably at least 90% identity, very especially preferably at least 95% identity, with a sequence of SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 over a length of at least 20, preferably at least 25, especially preferably at least 30, consecutive nucleotides, and very especially preferably over their full lengths.

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The degree of identity of the nucleic acid sequences is preferably determined with the aid of program GAP from the package GCG, Version 9.1, using standard settings.

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The sequences in accordance with the GenBank accession numbers (Acc. No.) AC002502, AF145639 and AC004420 are incorporated into the present description by reference.

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Subject-matter of the present invention are furthermore DNA constructs which comprise a nucleic acid according to the invention and a heterologous promoter.

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The term "heterologous promoter" as used in the present context refers to a promoter which has properties other than the promoter which controls the expression of the gene in question in the original organism. The term "promoter" as used in the present context generally refers to expression control sequences.

The choice of heterologous promoters depends on whether pro- or eukaryotic cells or cell-free systems are used for expression. Examples of heterologous promoters are the SV40, the adenovirus or the cytomegalovirus early or late promoter, the lac system, the trp system, the main operator and promoter regions of ^{phage} phage lambda, the fd coat protein control regions, the 3-phosphoglycerate kinase promoter, the acid phosphatase promoter and the yeast α -mating factor promoter.

Subject-matter of the present invention are furthermore vectors which contain a nucleic acid according to the invention or a DNA construct according to the invention. All the plasmids, phasmids, cosmids, YACs or artificial chromosomes used in molecular biology laboratories can be used as vectors.

Subject-matter of the present invention are also host cells comprising a nucleic acid according to the invention, a DNA construct according to the invention or a vector according to the invention.

The term "host cell" as used in the present context refers to cells which do not naturally comprise the nucleic acids according to the invention.

Suitable host cells are prokaryotic cells such as bacteria from the genera Bacillus, Pseudomonas, Streptomyces, Streptococcus, Staphylococcus, preferably E. coli, but also eukaryotic cells such as yeasts, mammalian cells, amphibian cells, insect cells or plant cells. Preferred eukaryotic host cells are HEK-293, Schneider S2, Spodoptera Sf9, Kc, CHO, COS1, COS7, HeLa, C127, 3T3 or BHK cells and, in particular, Xenopus oocytes.

Another subject-matter of the invention are antibodies which specifically bind to the abovementioned polypeptides or receptors. Such antibodies are produced in the customary manner. For example, such antibodies may be produced by injecting a substantially immunocompetent host with such an amount of a polypeptide according to the invention or a fragment thereof which is effective for antibody production, and

subsequently obtaining this antibody. Furthermore, an immortalized cell line which produces monoclonal antibodies may be obtained in a manner known per se. If appropriate, the antibodies may be labelled with a detection reagent. Preferred examples of such a detection reagent are enzymes, radiolabelled elements, fluorescent chemicals or biotin. Instead of the complete antibody, fragments may also be employed which have the desired specific binding properties. The term "antibodies" as used in the present context therefore also extends to parts of complete antibodies, such as Fa, F(ab')₂ or Fv fragments, which are still capable of binding to the epitopes of the polypeptides according to the invention.

The nucleic acids according to the invention can be used, in particular, for generating transgenic invertebrates. These may be employed in assay systems which are based on an expression, of the polypeptides according to the invention, which deviates from the wild type. Based on the information disclosed herein, it is furthermore possible to generate transgenic invertebrates where expression of the polypeptides according to the invention is altered owing to the modification of other genes or promoters.

The transgenic invertebrates are generated, for example, in the case of *Drosophila melanogaster*, by P-element-mediated gene transfer (Hay et al., 1997), or, in *Caenorhabditis elegans*, by transposon-mediated gene transfer (for example by Tc1; Plasterk, 1996).

Subject-matter of the invention are therefore also transgenic invertebrates which contain at least one of the nucleic acids according to the invention, preferably transgenic invertebrates of the species *Drosophila melanogaster* or *Caenorhabditis elegans*, and their transgenic progeny. The transgenic invertebrates preferably contain the polypeptides according to the invention in a form which deviates from the wild type.

Subject-matter of the present invention are furthermore processes for producing the polypeptides according to the invention. To produce the polypeptides encoded by the

5 nucleic acids according to the invention, host cells which contain one of the nucleic acids according to the invention can be cultured under suitable conditions, where the nucleic acid to be expressed may be adapted to the codon usage of the host cells. Thereupon, the desired polypeptides can be isolated from the cells or the culture medium in the customary manner. The polypeptides may also be produced in *in vitro* systems.

10 A rapid method of isolating the polypeptides according to the invention which are synthesized by host cells using a nucleic acid according to the invention starts with the expression of a fusion protein, it being possible for the fusion partner to be affinity-purified in a simple manner. For example, the fusion partner may be glutathione S-transferase. The fusion protein can then be purified on a glutathione affinity column. The fusion partner can then be removed by partial proteolytic cleavage, for example at
15 linkers between the fusion partner and the polypeptide according to the invention to be purified. The linker can be designed such that it includes target amino acids such as arginine and lysine residues, which define sites for trypsin cleavage. To generate such linkers, standard cloning methods using oligonucleotides may be employed.

20 Other purification methods which are possible are based on preparative electrophoresis, FPLC, HPLC (for example using gel filtration, reversed-phase or moderately hydrophobic columns), gel filtration, differential precipitation, ion-exchange chromatography and affinity chromatography.

25 Since GABA B receptors constitute membrane proteins, the purification methods preferably involve detergent extractions, for example using detergents which have no, or little, effect on the secondary and tertiary structures of the polypeptides, such as nonionic detergents.

30 The purification of the polypeptides according to the invention can encompass the isolation of membranes, starting from host cells which express the nucleic acids according to the invention. Such cells preferably express the polypeptides according to

the invention in a sufficiently high copy number, so that the polypeptide quantity in a membrane fraction is at least 10 times higher than that in comparable membranes of cells which naturally express GABA B receptors; especially preferably, the quantity is at least 100 times, very especially preferably at least 1000 times higher.

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The terms "isolation or purification" as used in the present context mean that the polypeptides according to the invention are separated from other proteins or other macromolecules of the cell or of the tissue. The protein content of a composition containing the polypeptides according to the invention is preferably at least 10 times, especially preferably at least 100 times, higher than in a host cell preparation.

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The polypeptides according to the invention may also be affinity-purified without a fusion partner with the aid of antibodies which bind to the polypeptides.

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Another subject-matter of the present invention are processes for the generation of the nucleic acids according to the invention. The nucleic acids according to the invention can be generated in the customary manner. For example, all of the nucleic acid molecules can be synthesized chemically, or else only short sections of the sequences according to the invention can be synthesized chemically, and such oligonucleotides can be radiolabelled or labelled with a fluorescent dye. The labelled oligonucleotides can be used for screening cDNA libraries generated starting from insect mRNA or for screening genomic libraries generated starting from insect genomic DNA. Clones which hybridize with the labelled oligonucleotides are chosen for isolating the DNA in question. After characterization of the DNA which has been isolated, the nucleic acids according to the invention are obtained in a simple manner.

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Alternatively, the nucleic acids according to the invention can also be generated by means of PCR methods using chemically synthesized oligonucleotides.

The term "oligonucleotide(s)" as used in the present context denotes DNA molecules composed of 10 to 50 nucleotides, preferably 15 to 30 nucleotides. They are synthesized chemically and can be used as probes.

5 The nucleic acids or polypeptides according to the invention allow new active compounds for crop protection and/or pharmaceutical active compounds for the treatment of humans and animals to be identified, such as chemical compounds which, being modulators, in particular agonists or antagonists, alter the properties of the GABA B receptors according to the invention. To this end, a recombinant DNA
10 molecule comprising at least one nucleic acid according to the invention is introduced into a suitable host cell. The host cell is grown in the presence of a compound or a sample comprising a variety of compounds under conditions which allow expression of the receptors according to the invention. A change in the receptor properties can be detected for example as described hereinbelow in Example 2. This allows, for example,
15 insecticidal substances to be found.

GABA B receptors alter the concentration of intracellular cAMP via interaction with G proteins, preferably after previously having been activated. Thus, changes in the receptor properties by chemical compounds can be measured after heterologous
20 expression, for example by measuring the intracellular cAMP concentrations directly via ELISA assay systems (Biomol, Hamburg, Germany) or RIA assay systems (NEN, Schwalbach, Germany) in HTS format. An indirect measurement of the cAMP concentration is possible with the aid of reporter genes (for example luciferase), whose expression depends on the cAMP concentration (Stratowa et al.,
25 1995). The coexpression of GABA B receptors with specific G proteins, for example $G\alpha 15$, $G\alpha 15$ or else chimeric G proteins, in heterologous systems and measuring the rise in calcium, for example using fluorescent dyes or equorin, is an alternative possibility of carrying out the screening (Stables et al., 1997; Conklin et al., 1993).

Furthermore, the binding of GTP to the activated G protein can be used as a read-out-system for assaying substances. Also, binding experiments with labelled GABA can be employed for screening.

5 The term "agonist" as used in the present context refers to a molecule which activates GABA B receptors.

The term "antagonist" as used in the present context refers to a molecule which displaces an agonist from its binding site.

10 The term "modulator" as used in the present invention constitutes the generic term for agonist and antagonist. Modulators can be small organochemical molecules, peptides or antibodies which bind to the polypeptides according to the invention. Other modulators may be small organochemical molecules, peptides or antibodies
15 which bind to a molecule which, in turn, binds to the polypeptides according to the invention, thus affecting their biological activity. Modulators may constitute mimetics of natural substrates and ligands.

20 The modulators are preferably small organochemical compounds.

The binding of the modulators to the polypeptides according to the invention can alter the cellular processes in a manner which leads to the death of the insects treated therewith.

25 The present invention therefore also extends to the use of modulators of the polypeptides according to the invention as insecticides.

30 The nucleic acids or polypeptides according to the invention also allow compounds to be found which bind to the receptors according to the invention. Again, they can be applied to plants as insecticides. For example, host cells which contain the nucleic acids according to the invention and which express the corresponding receptors or

polypeptides, or the gene products themselves, are brought into contact with a compound or a mixture of compounds under conditions which permit the interaction of at least one compound with the host cells, the receptors or the individual polypeptides.

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Using host cells or transgenic invertebrates which contain the nucleic acids according to the invention, it is also possible to find substances which alter receptor expression.

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The above-described nucleic acids according to the invention, vectors and regulatory regions can furthermore be used for finding genes which encode polypeptides which participate in the synthesis, in insects, of functionally similar GABA B receptors. Functionally similar receptors are to be understood as meaning in accordance with the present invention receptors which comprise polypeptides which, while differing from the amino acid sequence of the polypeptides described herein, essentially have the same functions.

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Information on the sequence listing and the figures

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SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5 show the nucleotide and amino acid sequences of the isolated GABA B cDNAs. SEQ ID NO: 2, SEQ ID NO: 4 and SEQ ID NO: 6 furthermore show the amino acid sequences of the proteins deduced from the GABA B cDNA sequences.

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Figure 1 shows a dose-effect curve of GABA and 3-APMPA on the *Drosophila* GABA B receptor composed of the polypeptides according to the invention with the amino acid sequences of SEQ ID NO: 2 and SEQ ID NO: 4, expressed in *Xenopus* oocytes.

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Figure 2 shows the functional coupling to the intracellular cAMP system of the coexpressed D-GABA B receptors R1/R2 composed of the polypeptides according to the invention with the amino acid sequences of SEQ ID NO: 2 and SEQ ID NO: 4.

- 5 HEK293 luc cells which have been stably transfected with D-GABA B R1/R2 (D-GABA R1/2) and untransfected control cells (control) were stimulated with forskolin, forskolin and GABA, and also with GABA alone, and the intracellular cAMP concentration was measured. The D-GABA B-R1/2-transfected cells showed a marked reduction in forskolin-induced cAMP response, while the control cells were unresponsive.

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Examples

Example 1

5 Isolation of the above-described polynucleotide sequences

Polynucleotides were manipulated by standard methods of recombinant DNA technology (Sambrook et al., 1989). Nucleotide and protein sequences were processed in terms of bioinformatics using the package GCG Version 9.1 (GCG Genetics Computer Group, Inc., Madison Wisconsin, USA).

Example 2

Generation of the expression constructs

The sequence regions of SEQ ID NO: 1, SEQ ID NO: 3 and SEQ ID NO: 5 were amplified by means of polymerase chain reaction (PCR) and cloned into the vector pcDNA3.1/Neo (Invitrogen, Groningen).

Heterologous expression

HEK293 cells were cultured at 5% CO₂ and 37°C in Dulbecco's modified Eagle's medium and 10% foetal calf serum. MBS (Stratagene, La Jolla, USA) was used for the gene transfer, following the manufacturer's instructions. 24 h to 48 h after the gene transfer, the cells were sown into microtiter plates at various densities. Recombinant cells were selected over 3 to 4 weeks by growth in Dulbecco's modified Eagles medium and 10% foetal calf serum and 700 µg/ml Geneticin (G418, Life Technologies, Karlsruhe) as selection marker. Individual resistant clones were analysed as described below.

Insect GABA B receptors were also expressed functionally in *Xenopus* oocytes. To this end, G-protein-activatable potassium channels (GIRK1 and GIRK4) were coexpressed in order to measure activation of the GABA B receptors (White et al., 1998).

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cAMP measurements

HEK293 cell strains were used for determining the cAMP concentration. On the one hand, HEK293 cells stably coexpressed the two *Drosophila melanogaster* receptors D-GABA B R1 and D-GABA B R2 (D-GABA R1/2). On the other hand, untransfected control cells were incorporated into the assay (control). In each case, the cells were plated into 96-well-plates at a density of 20,000 cells per cavity. Control cells were incubated in culture medium (DMEM, 10% FCS, penicillin and streptomycin, 50 U/ml and 50 µg/ml (Life Technologies)) and D-GABA-R1/2 expressing cells in selection medium (culture medium with 0.5 mg/ml Geneticin (G418, Life Technologies)) for 48 hours at 37°C until a cell density of approximately 80% was reached. Thereupon, the medium was removed, and the cells were washed once with unsupplemented DMEM. After incubation for 30 minutes with IBMX (300 µM) at 37°C, cells were stimulated for 30 minutes with GABA (100 µM) and/or forskolin (10 µM) at 37°C. All incubation steps were carried out in unsupplemented DMEM (Life Technologies). Then, the stimulation medium was removed and the cells were lysed with 50 µl of HCl (0.1 N) per cavity. The cells were lysed for 20 minutes at room temperature with shaking, and the cAMP concentration of the cell lysates were determined in triplicate using the enzyme immunoassay (EIA) kit AK-200 (Biomol, Hamburg, Germany) following the manufacturer's description.

Oocyte measurements

1. Oocyte preparation

5 The oocytes were obtained from an adult female *Xenopus laevis* frog (Horst Kähler, Hamburg, Germany). The frogs were kept in large tanks with circulating water at a water temperature of 20 - 24°C. Parts of the frog ovary were removed through a small incision in the abdomen (approx. 1 cm), with full anaesthesia. The ovary was then treated for approximately 140 minutes with 25 ml collagenase (type I, C-0130, SIGMA-ALDRICH CHEMIE GmbH, Deisenhofen, Germany; 355 U/ml, prepared with Barth's solution without calcium in mM: NaCl 88, KCl 1, MgSO₄ 0.82, NaHCO₃ 2.4, Tris/HCl 5, pH7.4), with constant shaking. Then, the oocytes were washed with Barth's solution without calcium. Only oocytes at maturity stage V (Dumont, 1972) were selected for the further treatment and transferred into microtiter plates (Nunc MicroWell™ plates, cat. No. 245128 + 263339 (lid), Nunc GmbH & Co. KG, Wiesbaden, Germany) filled with Barth's solution (in mM: NaCl 88, KCl 1, MgSO₄ 0.82, Ca(NO₃)₂ 0.33, CaCl₂ 0.41, NaHCO₃ 2.4, Tris/HCl 5, pH7.4) and gentamicin (gentamicin sulphate, G-3632, SIGMA-ALDRICH CHEMIE GmbH, Deisenhofen, Germany; 100 U/ml). Then, the oocytes were kept in a cooling incubator (type KB 53, WTB Binder Labortechnik GmbH, Tuttlingen, Germany) at 19.2°C.

2. Injecting the oocytes

25 Injection electrodes of diameter 10 - 15 µm were prepared using a pipette-drawing device (type L/M-3P-A, List-electronic, Darmstadt-Eberstadt, Germany). Prior to injection, aliquots with the D-GABA B DNA or GIRK1/4 DNA were defrosted and diluted with water to a final concentration of 10 ng/µl. The DNA samples were centrifuged for 120 seconds at 3200 g (type Biofuge 13, Heraeus Instruments GmbH, Hanau, Germany). An extended PE

5 tube was subsequently used as transfer tube to fill the pipettes from the rear end. The injection electrodes were attached to a X,Y,Z positioning system (treatment centre EP1090, isel-automation, Eiterfeld, Germany). With the aid of a Macintosh computer, the oocytes in the microtiter plate wells were approached, and approximately 50 nl of the DNA solution were injected into the oocytes by briefly applying a pressure (0.5-3.0 bar, 3-6 seconds).

3. Electrophysiological measurements

10 A two-electrode voltage terminal equipped with a TURBO TEC-10CD (npi electronic GmbH, Tamm, Germany) amplifier was used to carry out the electrophysiological measurements. The micropipettes required for this purpose were drawn in two movements from aluminium silicate glass (capillary tube, Article No. 14 630 29, l=100 mm, $\varnothing_{\text{ext.}}=1.60$ mm, $\varnothing_{\text{int.}}=1.22$ mm, Hilgenberg GmbH, Malsfeld, Germany) (Hamill et al., 1981).
15 Current and voltage electrodes had a diameter of 1-3 μm and were filled with 1.5 M KCl and 1.5 M potassium acetate. The pipettes had a capacitance of 0.2-0.5 MW. To carry out the electrophysiological measurements, the oocytes were transferred into a small chamber which was flushed continuously with normal Rimland solution (in mM: KCl 90, MgCl_2 3, HEPES 5, pH 7.2). To
20 apply a substance, the perfusion solution was exchanged for a substance solution with the same composition and additionally the desired substance concentration. The successful expression of the D-GABA B DNA was checked after one week at a terminal potential of -60 mV. Unresponsive oocytes were discarded. All the others were used for substance testing. The
25 data were documented by means of a YT plotter (YT plotter, Model BD 111, Kipp & Zonen Delft BV, AM Delft, Netherlands). When test substances were assayed in concentration series, these measurements were carried out on at least two different oocytes and at at least five different concentrations. The
30 substances have been assayed directly without preincubation in the presence of GABA (gamma-amino-N-butyric acid, A2129, SIGMA-ALDRICH

CHEMIE GmbH, Deisenhofen, Germany) for their antagonism. The individual data were entered in Origin (evaluation software Microcal Origin, Microcal Software, Inc., Northampton, MA 01060-4410 USA) (Additive GmbH, Friedrichsdorf/Ts, Germany). Means, standard deviation, IC₅₀ values and IC₅₀ curves were calculated using Origin. These measurements were carried out at least in duplicate.

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